A quantitative assessment of phosphorus forms in Australian soils

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Abstract
Solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy is by far the most widely-used spectroscopic technique for the speciation of soil organic P, but is yet to be used to characterise a wide range of Australian soils. Therefore, using this technique we analysed the NaOH-EDTA extracts of 18 diverse Australian soils. The majority of ^{31}P NMR signal was assigned to orthophosphate, representing 46 to 90% of total NaOH-EDTA extractable P. Orthophosphate diesters and pyrophosphate were present in all soil extracts, their concentrations ranging from 5 to 87 mg/kg (1-5% of total NaOH-EDTA extractable P) and up to 62 mg/kg (5% of total NaOH-EDTA extractable P) respectively. Up to 12 well-resolved orthophosphate monoesters resonances were identified (α- & β- glycerophosphate, *myo*-inositol hexa*kis*phosphate (phytate), adenosine-5´-monophosphate (AMP), *scyllo*-Inositol hexa*kis*phosphate). Orthophosphate monoesters were dominated by α- & β-glycerophosphate and phytate. All three compounds were assigned in all spectra with concentrations of α- & β glycerophosphate ranging from 1–5% and phytate up to 9% of total NaOH-EDTA extractable P. However, phytate concentrations were considerably lower than values determined previously for other soils. As well as numerous sharp resonances in the monoester region which we attributed to specific P-containing compounds, our results showed a large proportion of monoester P (24–65%) could be assigned to a single broad feature. We suggest that this broad signal is due to organic P found in large molecules such as humic acids.

Key Words
Organic phosphorus, ^{31}P NMR, Australian soils

Introduction
Phosphorus (P) is an essential nutrient required for plant growth. Although most soils contain large reserves of inorganic P, most of it is locked up in insoluble and tightly-bound forms. Organic P is not directly available to plants but can be converted into available inorganic P through hydrolysis or mineralization. The rate of P release from organic P forms depends partly on the specific organic P compounds present. Organic P contents vary appreciably among soils, but can represent up to 80% of total soil P (Dalal 1977). Australian soils are characteristically low in P for a variety of reasons. Perhaps most significantly, they are predominantly derived from sedimentary rocks, including sandstone, which are generally low in P. Additionally, over long periods of time these soils have experienced P losses due to leaching and erosion. Australian soils resistant to P deficiency are primarily those which occur in high rainfall zones where organic matter can accumulate (e.g. deep forested soils), or other areas where high iron (Fe) content has led to the retention of P by sesquioxides (Handreck 1997). However, not since Williams and Anderson (1968) has an attempt been made characterise the P forms in Australian soils. Therefore, the accurate characterisation of Australian soils will be useful in terms of aiding future efforts to access various P pools and manage P in agricultural systems (Cornish 2009a; b; Evans and Condon 2009; Guppy and McLaughlin 2009).

Solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy is by far the most widely-used technique for the speciation of soil organic P, the main reason being that of all the currently available techniques, it provides the most detailed and accurate information. In this study we use spiking experiments, and a modified method of spectral deconvolution to assess a wide range of Australian soils to provide a quantitative assessment of the P forms present.

Methods
NaOH-EDTA extraction
Eighteen soil samples were ground to pass through a 2-mm sieve prior to extraction. Soils were extracted in triplicate using the standard methods of Cade-Menun and Preston (1996). This involved shaking 2.0 g of soil with 40 mL of 0.25 *M* NaOH and 0.05 *M* Na_2EDTA for 16 h. The extracts were centrifuged (1400 x *g*) for 10

min and filtered using Whatman no. 42 filter paper. A 15 mL aliquot was immediately frozen and freeze-dried for NMR analysis. Triplicate sub-samples of the supernatant were also taken to determine the total P concentrations using nitric acid digestion and subsequent analysis by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

NMR analysis of NaOH-EDTA extracts
Triplicate freeze-dried NaOH-EDTA extracts for each soil were combined for NMR analysis. A 500 mg sub-sample of each composite extract was ground, re-dissolved in 5 mL of deionised water and centrifuged at 1400 *g* for 20 min. The supernatant solution (3.5 mL) and D_2O (0.3 mL) were placed in a 10 mm NMR tube. Solution ^{31}P NMR spectra were acquired at 24ºC on a Varian INOVA400 NMR spectrometer (Varian, Palo Alta, CA) at a ^{31}P frequency of 161.9 MHz. Recovery delays ranged from 10 to 30 s and were set to at least five times the T_1 value of the orthophosphate resonance determined in preliminary inversion-recovery experiments. We used a 90º pulse of 32 to 45 μs, an acquisition time of 1.0 s and broadband ^{1}H decoupling. Between 3224 and 49000 scans were acquired for each sample, depending on the P concentration of the freeze-dried extract. Chemical shifts were referenced to β-glycerophosphate at 4.63 ppm according to Doolette *et al.* (2009).

Quantification of P species for ^{31}P NMR spectra
Signal areas of classes of P compounds were calculated by integration by combining the spectral area occupied by the class of compound and the total P concentrations in the corresponding NaOH-EDTA extract. The four diagnostic chemical regions were orthophosphate (6.2–5.3 ppm), orthophosphate monoester (5.3–2.6 ppm), orthophosphate diester (2.0 to -1.0 ppm) and pyrophosphate (-4.5 to -5.5 ppm).

Individual peaks in the orthophosphate and orthophosphate monoester region were quantified by spectral deconvolution. Each spectrum was fitted with up to 15 sharp peaks (aromatic diesters, orthophosphate, α– and β–glycerophosphate, phytate, adenosine-5´-monosphosphate (AMP), *scyllo*-inositol phosphate and five unassigned peaks) and a broad base signal between 4 and 6 ppm.

Multiplying the relative intensity of the sharp peaks by the sum of the total extractable orthophosphate and monoester P concentrations gave the concentration of all P species in the soil. Phytate and orthophosphate concentrations were adjusted to account for the phytate C-2 peak which was obscured by the much larger orthophosphate peak. Total phytate concentration was calculated as 6/5 times the total concentration of the three observable resonances. Therefore, 1/5 of the total phytate concentration was subtracted from the total orthophosphate concentration.

Results
The assignment of the common peaks in each spectrum were identified by spiking known compounds into NaOH-EDTA extracts just prior to NMR analysis. Figure 1 shows the results of spiking experiments used to assign the resonances in the monoester region. It was clear that many of the peaks were common to most of the samples. These peaks included aromatic phosphate diesters, orthophosphate, α-glycerophosphate, phytate, β-glycerophosphate, AMP, *scyllo*-Inositol hexakisphosphate, orthophosphate diesters and pyrophosphate. All soils had an apparently low phytate concentration, which appears to contradict previous values determined using alternative methods.

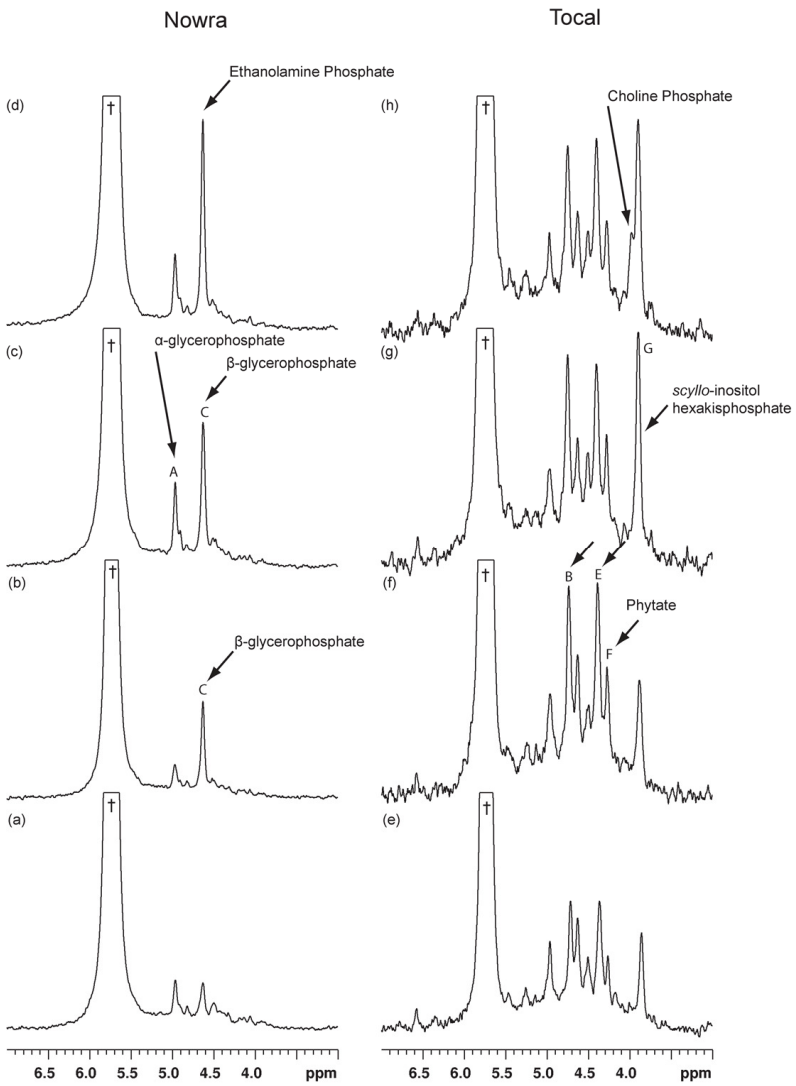

Figure 1. Solution ^{31}P nuclear magnetic resonance spectra of unspiked (a) Nowra and (e) Tocal NaOH–ethylenediaminetetraacetic acid (EDTA) soil extracts. The spectra show the consecutive addition of (b) β-glycerophosphate, (c) a 1:1 mixture of α- and β-glycerophosphate, and (d) ethanolamine phosphate to the Nowra NaOH–EDTA extract, and the consecutive addition of (f) phytate, (g) *scyllo*-inositol hexakisphosphate, and (h) choline phosphate to the Tocal NaOH–EDTA extract. Resonances A through G correspond to each of the peaks identified in the 18 soils. † Orthophosphate resonances. (reproduced (Doolette *et al.* 2009))

A large portion of organic P in the soil analysed was associated with a broad base signal between approx. 4 and 6 ppm. This complicates the deconvolution procedure and therefore the quantification of the spectra. This broad signal was present in all of the spectra, but was more easily discerned in the spectra which contained less intense monoester resonances. This broad base signal is overlooked in many other ^{31}P NMR spectra but has been identified in a few natural soils (Bünemann *et al.* 2008; Dougherty *et al.* 2007; Smernik and Dougherty 2007) but as observed by Bünemann *et al* (2008) and shown in Figure 2, is not observed in model soils that do not contain humified organic matter. It has been suggested that this broad feature may be due to large complexed forms of monoester-P in a wide range of only slightly different chemical environments, as opposed to smaller specific monoester-P compounds that form the sharp resonances (Dougherty *et al.* 2007).

Furthermore, it has been noted previously that failing to account for the broad signal can result in over-estimation of signal in the sharp resonances (Smernik and Dougherty 2007). Therefore, in providing a quantitative assessment of P in Australian soils it was vital to account for the presence of the broad signal in the monoester region, although it means a large portion of organic P cannot be assigned to specific molecules. This may also explain the surprisingly low phytate concentrations reported here, i.e. P which would have normally been assigned to phytate was assigned to the broad base signal.

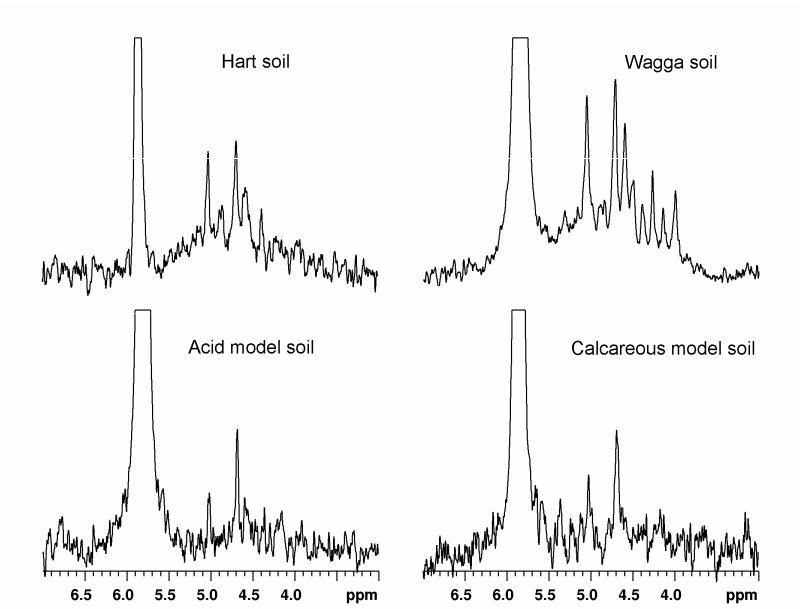

Figure 2. (Modified from Bünemann *et al* (2008)) solution ^{31}P NMR spectra of NaOH-EDTA extracts of a calcareous (Hart), acidic (Wagga) and acidic and calcarous model soil following a 25-week incubation with cellulose addition.

Conclusions

We have used an improved method for the speciation of soil P using solution ^{31}P NMR spectroscopy and spectral deconvolution. We showed that unless a broad, underlying signal in the orthophosphate monoester region is properly accounted for, the concentrations of other orthophosphate monoesters are likely to be inaccurately quantified. This broad signal represents a large portion of organic P, which we believe is P in large "humified" molecules. These findings have consequences for the way in which P cycling is currently viewed.

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